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Effect of Different Extraction Procedures on Antimicrobial Activity of Marine Bivalves: A Comparison

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ABSTRACT

Anti-bacterial activity was evaluated in different crude extracts of five commercially important edible marine bivalves, namely *Meretrix casta* (Chemnitz), *Polymesoda* (Geloina) *P. erosa* (Solander), *Perna viridis* (Linnaeus), *Crassostrea gryphoides* (Schlothim) and *Villorita cyprinoides* (Grey), collected from the coast of Goa (India). Three different procedures, *viz.* methanol (MeOH), PBS and acid-enzyme hydrolysis (AEH), were used to prepare the extracts. The efficacy of the extraction procedure was assessed on the antimicrobial activity. Antimicrobial assay was carried out against 8 bacterial strains (3 Gram positive and 5 Gram negative bacteria) and 1 species of fungi. The AEH extracts showed higher activity against the tested organisms as compared to MeOH and PBS extracts. The findings of the present study confirmed that the antimicrobial activity in bivalves appeared to be dependent on the extraction process. Considerable interspecies variation was also observed.

Keywords: Extraction procedures, methanol and PBS extracts, acid enzyme hydrolysate, antimicrobial peptides

INTRODUCTION

Due to an alarming rise in the occurrence of antibiotic resistant bacterial strains, the identification of new antimicrobial compounds has become one of the frontier areas in biomedical research. Marine invertebrates are known to rely on innate immune mechanisms which include both interacting cellular and humoral components to protect against potential pathogen (Tincu and Taylor, 2004). Innate immune mechanism in marine invertebrates is known to protect these organisms against potential pathogens. Moreover, it has been well known that the innate immunity is triggered immediately after microbial infection to produce antimicrobial compounds including small antimicrobial peptides (AMP). In recent years, it has widely been recognized that AMPs are strong defensive weapons against bacteria and/or fungi, viruses, or parasites in multicellular organisms (Zasloff, 2002). Furthermore, AMPs are also known as major components of innate immune defence system in invertebrates (Seo *et al.*, 2005).

Considering the fact that the marine animals can survive in a hostile environment where they are surrounded by various pathogenic organisms, including human pathogens (Bouchriti and Goyal, 1992) and that they are potential sources for bioactive compounds, an attempt was done in the present study to evaluate the antimicrobial activity in five commonly occurring edible bivalves, such as *Meretrix casta* (Chemnitz), *Polymesoda* (Geloina) *P. erosa* (Solander), *Perna viridis* (Linnaeus), *Crassostrea gryphoides* (Schlothim) and *Villorita cyprinoides* (Grey). In

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continuation with the same effort, an attempt was also made to assess and compare the efficacy of the extracts prepared using three different extraction procedures.

MATERIALS AND METHODS

Live bivalves used in the present study were collected from various beaches of Goa, Maharashtra and Karnataka in India. These bivalves were not collected during the summer months to avoid stress related to disease, elevated water temperature, hypoxia or gametogenesis.

Bivalves were brought to the laboratory in seawater, washed, and de-shelled; tissue and mantle fluids were also collected. Material collected from each animal was divided into three equal parts for the preparation of Methanol (MeOH), phosphate buffer saline (PBS) extracts with protease inhibitors and acid enzyme hydrolysate (Chatterji *et al.*, 2000). These procedures yielded four different types of extract from each animal. They were designated as MeOH, PBS, AEH -MeOH and AEH- Aq extracts.

All the extracts were quantitatively analyzed using the standard methods for the estimation of total protein (Lowry *et al.*, 1951), carbohydrates (Dubois *et al.*, 1956) and lipids (Parsons *et al.*, 1984).

The antimicrobial activity of the bivalve extracts prepared was evaluated against a set of 9 pathogenic micro-organisms (*Bacillus subtilis, Escherichia coli, Pseudomonas* sp, *Streptococcus pyrogenes, Staphylococcus aureus, Proteus vulgaris, Klebsiella pneumoneae, Serrratia marganii* and *Candida albicans*) using standard disc diffusion assay (qualitative) and liquid growth inhibition assay (quantitative). The liquid growth inhibition assay was carried out at 32±1°C for 30 hrs. Three sets of sterilized glass vials, with 2.5 ml nutrient broth (NB), were prepared for each pathogenic micro-organism. These sets were inoculated with one bacterial suspension (18 hr old), and out of which, one set each was inoculated with 10 µl of Gentamycin (1 mg/ml). This was treated as the positive control. In another set, all the micro-organisms were inoculated with 10 µl of animal extracts (50 mg/ml). One set was maintained as the negative control to see the normal growth of the tested organisms. After 2 hrs, an aliquot was taken in a micro titer plate and optical density was measured at 570 nm using an ELISA reader (BioRad, Microplate Reader, Model No. 680). The percentage of the inhibition growth was calculated with absorbance values using the following equation, where CI is the percentage Inhibition index, A = A_{570} of bacteria with NB (control) and B = A_{570} of bacteria with extract or Gentamycin (experimental).

$$C\,I = \left[\frac{A - B}{A}\right] \times 100$$

RESULTS

The total protein content was the highest as compared to the total contents of carbohydrate and lipids in all the extracts. The total content of lipid in all the extracts was very less as compared to the total protein and carbohydrate (Table 1).

Bioassay of MeOH extracts, using standard disc diffusion assay, showed that *P. erosa* appeared to be the most active one against seven tested organisms. In case of the PBS extracts, *M. casta* showed the highest antimicrobial activity against all pathogens. Meanwhile, in the case of AEH-MeOH extract, significant antimicrobial activity was shown by *C. gryphoides* against all the pathogens. On the other hand, the AEH-Aq extracts of *P. erosa* showed a very good activity against all the tested organisms (Table 2).

As compared to Gentamycin, all the MeOH extracts showed more than 30 to 50% antimicrobial activity against *E. coli* when liquid growth inhibition assay was performed. The MeOH extract of *P. erosa* appeared to be the most promising extract showing antimicrobial activity against both Gram positive and Gram negative tested organisms for 18-24 hrs (*Fig. 1*). The activity of the extract of *M. casta* PBS was better than Gentamycin for 10 hrs. It was interesting to note that Gentamycin did not have very good antimicrobial activity against *S. marganii*, but all the PBS extracts (except *V. cyprinoides*) were able to inhibit the growth (>50%) of Gram negative bacterium for more than 6 hrs (*Fig. 1*). In the case of AEH–MeOH extracts, it was observed that *C. gryphoides, V. cyprinoides, M. casta* and *P. erosa* appeared to be better than or at par with Gentamycin in successfully inhibiting the growth of some pathogens (*Fig. 1*). In the group of AEH–Aq extracts, different extracts (except *V. cyprinoides*) inhibited the growth of *E. coli, K. pneumoneae, S. marganii* and *S. aureus* better than Gentamycin for 4–10 hrs (*Fig. 1*).

TABLE 1
Biochemical analysis of different bivalves

Extracts		Biochemical analysis		
		Protein (µg/ml/mg)	Carbohydrate (µg/ml/mg)	Lipid (µg/ml/mg)
P. erosa	MeOH	420	24.48	0.6
	PBS	640	2.31	0.07
	AEH-MeOH	528	7.08	0.19
	AEH-Aq	154	6.54	0.02
P. viridis	MeOH	280	24.72	0.09
	PBS	830	32.05	0.04
	AEH-MeOH	440	2.04	0.05
	AEH-Aq	170	1.05	0.01
M. casta	MeOH	190	5.76	0.15
	PBS	960	24	0.08
	AEH-MeOH	356	7.2	0.03
	AEH-Aq	194	32.77	0.01
C. gryphoides	MeOH	270	27.61	0.2
	PBS	370	15	0.07
	AEH-MeOH	744	6.48	0.03
	AEH-Aq	126	16.68	0.02
V. cyprinoides	MeOH	246	34.28	0.15
	PBS	415	19.58	0.05
	AEH-MeOH	720	22.08	0.06
	AEH-Aq	176	5.25	0.02

All the experiments were carried out in triplicates and the results are expressed as mean values. The results were then compared using the two-way ANOVA test (StatSoft, 1999). p values < 0.05 were considered as significant. In this study, it was observed that when the antimicrobial activity of one extract was compared to all the tested organisms, the p value < 0.05 indicated that the presence of antimicrobial compound/ peptide in the extract could have different degrees of activity against various organisms.

DISCUSSION

Most of the work carried out on antimicrobial compounds from marine bivalves deal with *M. edulis, M. galloprovicialis, G. demissa, C. verginica* and *C.* gigas (Haug *et al.*, 2004; Tincu and Taylor, 2004; Seo *et al.*, 2005). In this study, an attempt was done to screen a few more bivalves, especially the commonly occurring edible ones. The source for majority of the AMPs reported has been from the hemocytes (Charlet *et al.*, 1996; Mitta *et al.,* 1999a,b), epithelial tissues (Marshall and Arenas, 2003; Noriaki *et al., 2003*) and the tissues of gut and respiratory organs (Tincu and Taylor, 2004). Considering this an important aspect, the extracts were prepared using both mantle tissue and mantle fluid of the bivalves.

Presuming that the antimicrobial compounds were either protein or peptide in nature, a combination of 'soft techniques' was selected for the preparation of crude Methanol (homogenization with chilled Methanol + filtration) and PBS extracts (homogenization with PBS + protease inhibitor + centrifugation) to ensure that the functionality and/or the biological activity of the analytes remained intact (Visser *et al.,* 2005). For this, methanol

TABLE 2
Antimicrobial activity (zone of inhibition in mm) induced by different extracts of five bivalves

Test Organism	*P. erosa*				*P. viridis*				*M. casta*				*C. gryphoides*				*V. cyprinoides*			
	Me OH	PBS	AEH MeOH	AEH Aq	Me OH	PBS	AEH MeOH	AEH Aq	Me OH	PBS	AEH MeOH	AEH Aq	Me OH	PBS	AEH MeOH	AEH Aq	Me OH	PBS	AEH MeOH	AEH Aq
S. aureus	11	9	8	6	-	6	-	6	11	8	8	9	10	-	6	12	-	-	8	7
S. pyrogenes	12	8	7	11	11	9	-	7	7	8	6	7	11	9	12	-	-	-	9	-
B. subtilis	13	7	12	12	-	8	-	-	-	12	7	-	9	-	6	-	-	-	-	11
E. coli	8	-	11	12	9	-	-	10	6	11	13	6	9	-	7	12	-	-	7	9
Pseud. sp	12	-	10	11	10	6	6	-	-	7	11	-	-	9	8	-	-	-	9	11
S. marganii	11	8	-	6	-	6	6	6	12	7	-	6	-	9	13	-	-	-	-	11
P. vulgaris	-	-	10	12	-	-	-	7	6	6	-	7	-	-	12	7	-	-	-	13
K. pneumon.	-	6	9	7	-	6	-	6	6	12	7	6	-	7	9	7	-	9	9	12
C. albicans	10	6	6	12	-	10	7	7	-	11	9	10	-	12	8	10	-	-	9	13

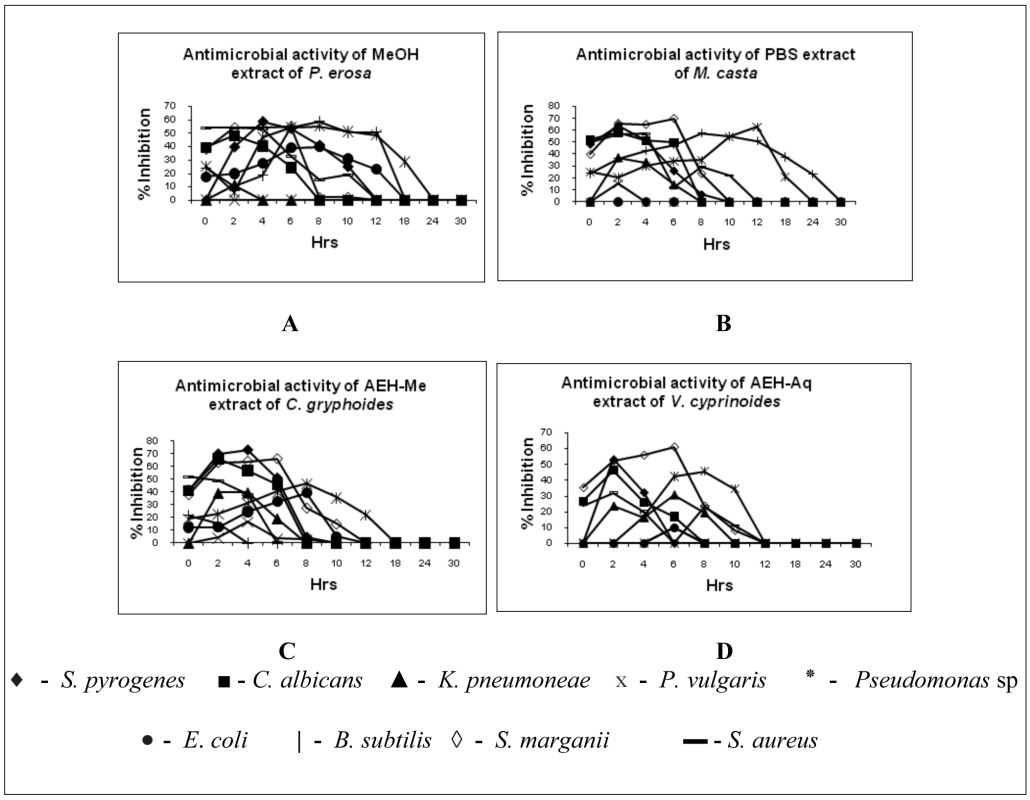

Fig. 1: Antimicrobial activity induced by different extracts against pathogenic organisms [Best activity of *Gentamycin against* - S. pyrogenes - 73% (10 h), C. albicans - 74.5% (10 h), K. pneumoneae - 28% (10 h), P. vulgaris - 48% (2 h), Pseudomonas *sp* - 63.5% (10 h), E. coli - 8.5% (0 h), B. subtilis - 78% (30 h), S. marganii - 39% (30 h), S. aureus - 69% (10 h)]

was selected as a suitable solvent as it gave good extraction efficiency. Most of the low molecular weight proteins/peptides (stable at room temperature) were extracted from it and had an added advantage of allowing rapid sample concentration through evaporation. The other solvent/homogenization medium is PBS as most of the active high molecular weight proteins and peptides are known to get extracted in it. Protease inhibitor simultaneously deactivated proteolytic enzymes in the tissue which would otherwise cause rapid degradation of the proteins/peptides (Conlon, 2007). The results of antimicrobial assay in the present study indicated that these extracts showed high antimicrobial activity against the tested pathogens, indicating that these procedures are capable of extracting the antimicrobial compound(s), with relatively higher activity, without degrading the nature of the compound.

Extraction from a biological matrix can also be achieved using the 'harsh' techniques in which extraction conditions deviate from physiological conditions and result in completely different physico-chemical properties of the analytes and interfering molecules. The AEH extraction procedure employed in the present study was very harsh as all the conditions (e.g. highly acidic pH, 100°C temperature) were totally different from the physiological conditions of the biological matrices. Enzyme protosubtilin hydrolyzed high-molecular animal proteins to short peptides and a mixture of free amino acids. The crude extract has been reported to possess short peptides, free amino acids (conjugated with metals like Cu, Zn etc) and minerals. They were also reported to possess low fat and salt contents (Chatterji *et al.*, 2000). The antimicrobial activity of the AEH extract might be either due to short peptides, amino acids conjugated with metal ions

or both or it could also be due to the generation of artefacts during the extraction process.

The standard disc diffusion method is a sensitive and highly accepted method used for the detection of antimicrobial activity, but many feel that it is a qualitative method and should not be used to quantify the activity (Rios *et al.*, 1988). Therefore, the results were quantified using the liquid growth inhibition assay. These results largely confirmed the findings of disc diffusion assay and helped in calculating the inhibition percentage of culture growth caused by a particular extract towards the pathogenic organism(s). This assay was carried out for 30 hrs to compare the time when the extracts started inhibiting the growth of the organism and when it lost the activity. It was observed that most of the crude extracts were able to induce inhibition for ±10 hrs and also showed antimicrobial activity at par (or better at some places) with a purified antibiotic like Gentamycin.

The results presented in this paper are the first stage of a bioassay-based baseline survey to achieve the isolation, purification, structure elucidation and biological testing of the active compound(s) from the potent marine bivalves. This comparative data suggests that *P. erosa, M. casta,* C. *gryphoides* and *V. cyprinoides* are the potential candidates for the isolation and purification of potent antibiotics. Moreover, the extraction of the antimicrobial compound(s) appeared to be dependent on the extraction procedure and the nature of the solvents used for extraction.

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